

The University of Chicago

THE RELATION BETWEEN VITAMINS
AND THE GROWTH AND SURVIVAL
OF GOLDFISHES IN HOMOTYPI-
CALLY CONDITIONED WATER

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

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GERTRUDE EVANS

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THE RELATION BETWEEN VITAMINS AND THE GROWTH AND SURVIVAL OF GOLDFISHES IN HOMOTYPICALLY CONDITIONED WATER ¹

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FOUR FIGURES

INTRODUCTION

Recent work in this laboratory has shown that fishes isolated in water in which other fishes of the same species have lived, grow faster than those isolated in similar water which has not been biologically conditioned (Allee et al., '34, '36). An analysis of the factor or factors to be found in such homotypically conditioned water required an investigation of the possibility that it contained some accessory food factor contributed by the conditioning fishes which was not present in sufficient amounts in the diet of the experimental fishes to give a maximum effect on growth or survival or both. It is well known that rats are able to maintain growth on an otherwise inadequate diet if allowed access to their excreta (Steenbock, Sell and Nelson, '23). The significance of this fact had been recognized early in the history of vitamin research. It seemed possible that in a similar way, the favorable effect of conditioning the water might be due to the presence of vitamins excreted by the conditioning fishes. In this case the entire medium in which the fishes live is affected by excretory products.

¹ I am greatly indebted to Prof. W. C. Allee for suggesting this problem and for his interest and generous aid throughout the course of these studies. This work was financially supported in part by a grant from the Rockefeller Foundation to aid investigations in the biological sciences at The University of Chicago.

The value of certain vitamins for fish has been indicated by various workers: vitamins A, B and C for carp and possibly trout (Davis and James, '24); vitamin B for rainbow trout (Haempel and Peter, '32); vitamins D and possibly G for goldfish and channel cat (Jewell, Schneberger and Ross, '33); the vitamins of yeast and cod liver oil for rainbow trout (Davis, '27) and for brook trout (McCay et al., '30). McCay and Dilley ('27) postulated some factor in raw meat which is not vitamin A, B, C, D or E as the major factor in growth and called it 'factor H.' The properties of 'factor H' have been further investigated by McCay and his co-workers (see Literature Cited). It was found to be totally destroyed in cooked dried meats and only partially preserved in an alcoholic extract of fresh liver and vacuum dried liver. Over extended periods of time, according to McCay, the needs of the fish for 'factor H' greatly complicate any critical tests on vitamin requirements since thus far 'factor H' has not been isolated and adding whole meat to the diet adds other vitamins as well.

With this background for reference, experiments were devised in an attempt to determine whether the conditioning agent in our fish experiments might be a vitamin factor. An analysis of the problem involved four different sets of conditions: fishes fed on, 1) a vitamin-rich diet in conditioned water (series CR); 2) the same diet in non-conditioned water (NR); 3) a vitamin-free diet in conditioned water (CF), and 4) the same diet in non-conditioned water (NF).

MATERIAL AND METHODS

The experiments to be reported were all made on the common goldfish, *Carassius auratus* L.² The technique used was developed in previous experiments on fish growth in conditioned water by other workers in this laboratory and is recorded at some length elsewhere (Allee et al., '34). Briefly this technique was as follows. Synthetic pond water was

² We take this opportunity to acknowledge with thanks the generosity of the Grassyfork Fisheries, Inc., of Martinsville, Indiana, in providing all of the fish used in these experiments, without cost.

made up daily with analyzed distilled water from a still of block tin, to contain salts in the following proportions: CaCl_2 , 100 mg.; NaNO_3 , 50 mg.; MgSO_4 , 50 mg.; K_2SO_4 , 50 mg. per liter. The salts used were Baker and Adamson quality 'Reagent' calcium chloride and potassium sulphate, and Merck's 'Reagent' magnesium sulphate and sodium nitrate. The concentrated solutions used contained no heavy metals according to the standard HCl test. In such synthetic pond water there was no change in the total amount of electrolytes over 22-hour periods of goldfish conditioning (Allee et al., '34). The water was prepared daily and drawn off into two large glass tanks; in one a number of fishes were placed to condition the water and the other was kept as control. This water was used for the assay fishes on the following day.

The assay fishes, ranging from 25 to 33 mm. in length from anterior margin of eye to base of tail, were isolated in separate glass containers in 2 liters of water with the exception of one experiment in which with smaller fishes, 1.7 liters of water were used. At the beginning and end of the experimental periods (20 to 30 days) they were photographed by the method previously described by Allee et al. ('34). The assay fishes were fed for 2 to 3 hours each day and were transferred at the end of this time to a second set of containers which had been filled with conditioned or non-conditioned water as required by the experiment. The conditioning fishes were fed for 2 hours also in a separate tank and were washed and returned to the conditioning tank with fresh water at the end of the feeding period.

The amount of growth was determined by measuring the photographs taken at the beginning and end of the assay period. Growth was recorded as increase or decrease in length plus width; this seemed to be a more accurate representation of the condition of the fishes than measurements of growth in length alone. In fishes that were growing very little, or not at all, there was frequently a decrease in width measurements. An actual decrease in length was also sometimes observed. The standard error of the combined measurements of length plus width is $\pm 0.06\%$.

COMPOSITION OF THE DIETS

The diets used for comparison were selected so as to provide on the one hand, an adequate diet rich in vitamins, and on the other, a diet comparable in starch, protein and salt content, but deficient in vitamins. In a preliminary series of ten experiments the vitamin-rich diets were composed of natural foodstuffs supplemented with raw liver and vitamin containing substances, and the vitamin-free diets, of purified products. While efforts were made to have the carbohydrate-protein ratio and caloric content equivalent in the vitamin-rich and vitamin-free foods, these diets may be criticized for their difference in consistency and for a possible difference in palatability. Moreover, the addition of raw meat to the vitamin-rich foods must have provided factors other than vitamins which were without proper controls in the vitamin-free diet. Therefore only the most critical experiments are reported here, in which a basal diet³ of highly purified products was used and vitamin containing substances were added for the vitamin-rich diets (table 1). Raw liver was omitted entirely. For the corresponding vitamin-free diets, except as noted later, Wesson salad oil was added to the basal mixture, equivalent to the amount of haliver oil in the vitamin-rich diet. Water was added as control for the lemon juice. No addition was made as a control for the yeast.

In experiment 11 the dry materials were mixed thoroughly and added to a gelatin solution which was prepared daily by soaking the gelatin in 5 cc. of cold water and adding the remainder of the liquid as boiling water. Lemon juice and haliver oil were added to the vitamin-rich diet at this stage. The mixture was allowed to gel, then diced and fed to the fishes.

In experiment 12 the basal diet (V) differed from that of experiment 11 (diet H) by the use of 'vitamin-free casein.' This casein was granular and although the diets were prepared in the same way as in the previous experiment, the

³ Dr. Lydia J. Roberts of the Department of Home Economics and Household Administration gave valuable assistance in planning these diets.

mixture did not adhere so well and growth was poor. For the second 20-day period in this experiment a return was made to the former kind of casein (diet H) and better growth resulted.

In experiment 14 the 'vitamin-free casein' was used in larger proportions and cooked starch served as a binder in place of the gelatin previously used. (Cooked starch has

TABLE 1
Composition of the vitamin-rich diets

EXPERIMENT	DIET ¹	TIME USED	CASEIN ²	STARCH ³	SALTS ⁴	HALIVER OIL ⁵	YEAST ⁶	LEMON JUICE ⁷	DIS-TILLED WATER	GELATIN
		<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>drops</i>	<i>gm.</i>	<i>cc.</i>	<i>cc.</i>	<i>gm.</i>
11	H+	60	3.5 (H)	5.1	0.4	10	1	4	15	1
12	V+	20	3.7 (V)	5.1	0.4	10	1	4	15	1
	H+	20	3.5 (H)	5.1	0.4	10	1	4	15	1
14	C+	60	5.1 (V)	4.5	0.4	10	1	4	24	..
15	C+	20	5.1 (V)	4.5	0.4	10	1	4 ⁸	24	..
	C+	20	5.1 (V)	4.5	0.4	6	1	4	24	..
	C+	60	5.1 (V)	4.5	0.4	1	1	4	24	..
	C+	40	5.1 (V)	4.5	0.4	4	1	4	24	..

¹ The corresponding vitamin-free diets are designated elsewhere as H, V, C.

² Casein (H)—Merck, according to Hammarsten; (V) vitamin-free casein of Casein Manufacturing Company of America.

³ Potato starch.

⁴ Osborne and Mendel salt mixture (Osborne and Mendel, '19).

⁵ Haliver oil (Parke Davis, haliver oil with viosterol, 250 D) for vitamins A and D.

⁶ Dried brewers' yeast powder in experiments 11 and 12. Pure dehydrated yeast (Northwestern Yeast Co.) in experiments 14 and 15 for vitamins B and G. I am indebted to Dr. M. H. Givens of the Northwestern Yeast Co., through whose courtesy pure dehydrated yeast was provided without cost.

⁷ Fresh lemon juice for vitamin C.

⁸ Lemon juice was cooked with diet after 10 days in experiment 15.

been found to be more favorable in a fish diet than raw starch. Titcomb et al., '29.) This diet gave satisfactory results in growth. In preparing the diets daily, the dry mixture and water were cooked for about 3 minutes to form a thick paste or dough. The vitamin containing substances, haliver oil, yeast and lemon juice, were then worked into the

dough; the food was cut up and a portion dropped into each fish container.

In experiment 15 the same dry mixture was used as in experiment 14. The preparation of the diets was changed after the first 10 days by adding the lemon juice before cooking. According to Sherman ('27), cooking for such a short time (3 minutes) should not destroy much of the vitamin C present in lemon juice. The amount of haliver oil was reduced during the later experimental periods. (There was evidence from rat experiments of the extreme potency of haliver oil in vitamins A and D.) After 40 days, because of the small amount used, Wesson oil was omitted as a control in the vitamin-free diet.

CONDITIONED WATER AND CONDITIONING FISHES

For conditioning the water different numbers of conditioning fishes were used, depending on the size of the fishes and the amount of water. The conditioning coefficient, expressed as the average length of the conditioning fishes in millimeters times the number of fishes, divided by the number of liters of water to be conditioned ($\frac{L}{V}$) ranged from 20 to 25 in most of the experiments. The conditioning fishes were fed on the same vitamin-rich diet as the experimental fishes in the preliminary experiments. In experiments 11, 12 and 14 they were fed on raw liver and oatmeal, and the conditioning water was not sieved since it had been shown by other workers that this treatment did not radically change the conditioning effect. Only in experiment 15 was the synthetic vitamin-rich diet fed to the conditioning fishes and as a final check the conditioned water was all strained through fine phospho-bronze sieves with an average opening of 0.04 mm. It is probable that the different types of diets affected the character of the faeces and regurgitated food, and thus of the water, although this material was removed insofar as was possible by sieving in the last experiment. In general, the same conditioning fishes were used throughout an experiment. In experiment 15 which lasted for 140 days, ten of

the original fifteen conditioning fishes were removed during the course of the experiment and replaced by three new fishes. The amount of water was correspondingly reduced to maintain the same conditioning coefficient. Occasionally during the course of the other experiments also, a conditioning fish infected with fungus was replaced by a healthy fish of approximately the same size.

EXPERIMENTAL RESULTS

The results of the preliminary experiments will not be given in detail here; an extended account has been deposited as a bound manuscript in the library of The University of Chicago (Evans, '36). In general, they bear out with few exceptions, the favorable effect of conditioned water described by other workers. The effects of vitamin-rich and vitamin-free diets were less consistent. Four experiments in conditioned water gave an average difference of 2.75% greater growth on the vitamin-rich than on the vitamin-free diet, with a statistical probability⁴ of 0.0267, while six experiments in non-conditioned water gave practically no difference. The results from the series in conditioned water suggest an effect of the lack of vitamins. However, the difference in growth on the two diets in these preliminary experiments cannot be considered as positive evidence of a vitamin effect, in view of the known difference in constituents and in physical consistency of the two diets. Even supposing an exactly equivalent food value of the two types of diets, the observed effects might still be due to the presence of the hypothetical 'factor H' found in raw liver.

The pertinent data and growth results of the critical experiments at the end of 20 days are given in table 2. Experiment 12 was continued for 40 days, experiments 11 and 14 for 60 days, and experiment 15 for 140 days with measure-

⁴Statistical significance is based on Student's method. A value of 0.05 represents five chances in 100 of getting as great a deviation by random sampling as the one observed. This corresponds to three times the probable error of the mean difference and is considered the upper limit of statistical significance; the smaller this fraction, the greater the significance.

ments made at 20-day intervals. In no case did the relationships established between the different series of assay fishes during the first 20 days with regard to growth differ in the later periods of the experiments. The number of fishes surviving in each series after extended periods of experimentation are the most significant data of these later periods;

TABLE 2
Growth results

EXPERI- MENT NO.	GENERAL CONDITIONS							
	Number of fish	Average initial L. + W. ¹	Cond. coefficient	Volume (liters)	Sieved	Days	Diet	
11	40	55.8	23	2	—	20	H+	H
12	40	54.6	23	2	—	20	V+	V
14	40	54.5	23	2	—	20	C+	C
15	48	43.5	24-25	1.7	+	20	C+	C

¹Length plus width in millimeters measured on photographs. To get actual values in millimeters multiply by factor 0.82.

EXPERI- MENT NO.	RESULTS									
	Per cent increase in L. + W.				Statistical probability					
	CR ²	CF ³	NR ⁴	NF ⁵	CR-NR	CF-NF	CR-CF	NR-NF	CF-NR	CR-NF
11	+3.2	+3.0	-3.4	-3.1	0.0004	0.0010	0.8438	0.7686	0.0018	0.0002
12	-0.7	-2.2	-4.3	-5.5	0.0036	0.0012	0.0908	0.1806	0.0438	0.0000
14	+6.2	+4.6	+3.0	+3.4	0.0510	0.3806	0.4348	0.7678	0.2466	0.0896
15	+2.7	+2.7	-0.9	-0.7	0.0146	0.0168	1.0000	0.8436	0.0142	0.0138

²CR, conditioned water, vitamin-rich diet.

³CF, conditioned water, vitamin-free diet.

⁴NR, non-conditioned water, vitamin-rich diet.

⁵NF, non-conditioned water, vitamin-free diet.

these results are given in table 4. These extended experiments test fairly adequately for a possible delayed effect of a vitamin deficiency which would not be apparent in shorter tests.

A study of table 2 shows that in all four experiments the fishes in conditioned water grew more than those in non-conditioned water and that in all but experiment 14, the differences are statistically significant. In experiment 14

there was more than usual variation in the amount of growth of the individual fishes. The fact that these fishes grew well in non-conditioned water also lessens the statistical significance but serves to demonstrate the adequacy of the whole experimental procedure and of the diet. These fishes were used very soon after receiving them from the hatchery, and they may have been in unusually good condition for growth. The fishes on the vitamin-rich diet in conditioned water grew 6.2% as opposed to 4.6% on the vitamin-free diet, but the difference is statistically insignificant, as is the difference between the growth of 3.0% and 3.4% on the two diets in non-conditioned water. In experiments 11, 12 and 15 there is a striking uniformity in growth of fishes on the vitamin-rich and vitamin-free diets in any one experiment. In experiment 11 the fishes grew 3.2% and 3.0% on the vitamin-rich and vitamin-free diets in conditioned water, and decreased in size 3.4% and 3.1% in non-conditioned water. In experiment 15 these values were 2.7% for both diets in conditioned water and -0.9% and -0.7% for the two diets in non-conditioned water. While the fishes failed to grow in any series in experiment 12, there was greater failure in non-conditioned water, and the differences between the series fed on the two diets were insignificant.

The similarity of growth on the two diets is again shown in figure 1 which summarizes the results for the four series of experiment 15, taken at 20-day intervals. During the later experimental periods (from 60 days on) the growth rates were very slow and there was a high percentage of deaths. It was noticed that none of the fishes were eating their food well, and particularly in non-conditioned water the food seemed untouched. In other animals loss of appetite is frequently evidence of a dietary deficiency, and in this case might be due to a lack of fresh meat in the diet since vitamins A, B, C, D and G were all provided. Even the conditioning fishes were perceptibly thinner; of the original fifteen fishes two died and seven unhealthy fishes were removed during the 140 days of the experiment.

Table 3 gives the statistical analysis of the growth of fishes in the four series averaged for the four critical experiments. Good growth resulted on both diets in conditioned water. This averaged 2.8% for the vitamin-rich diet at 20 days and

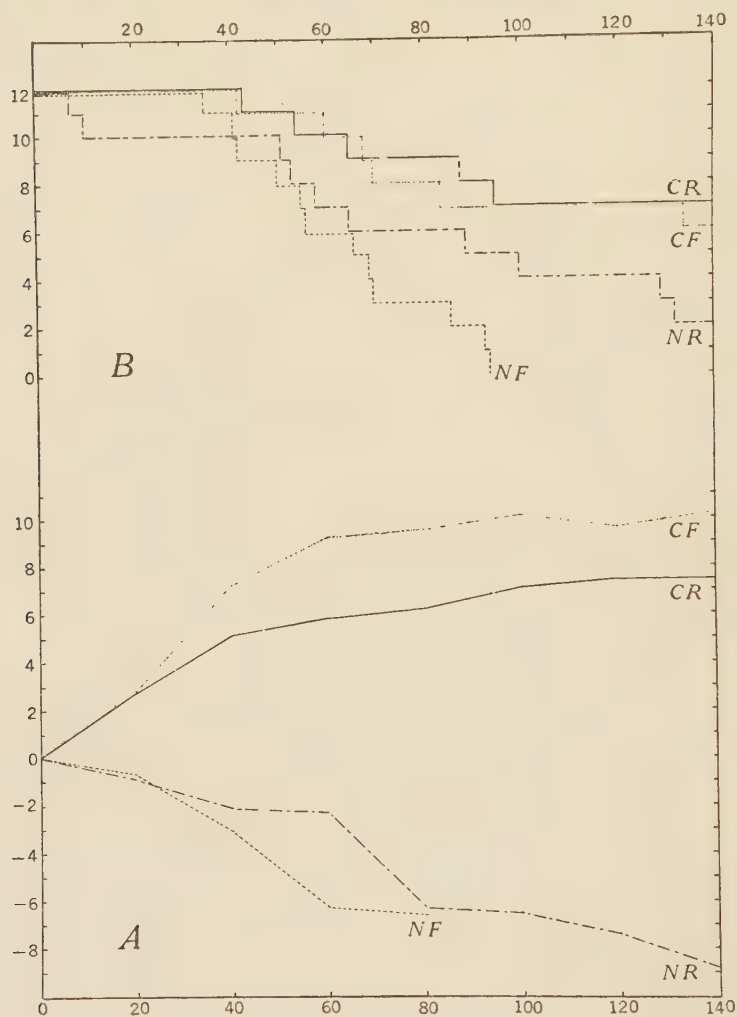


Fig. 1 Growth and survival of fishes in experiment 15. A, growth; B, survival. The horizontal axis gives time in days. The vertical axis represents percentage increase or decrease in length plus width for the growth curves; and numbers of fishes surviving for the survival curves. CR, conditioned water, vitamin-rich diet; CF, conditioned water, vitamin-free diet; NR, non-conditioned water, vitamin-rich diet; NF, non-conditioned water, vitamin-free diet.

2.02% for the vitamin-free, with an average difference of 0.83%. This has a statistical probability of 0.1294 and is an insignificant difference, indicating that there is no vitamin effect on growth. While this fact contradicts the evidence from the preliminary group of experiments, these last experiments are more critical. Failure in growth occurred in non-conditioned water on both diets, as in the earlier experiments, and again the difference between the results for the two diets was statistically insignificant.

From these relationships there resulted a significant difference between the effect of conditioned and non-conditioned

TABLE 3

Summary of growth data—statistical comparisons of experiments 11 to 15 at 20 days

COMPARISON OF SERIES	PERCENTAGE GROWTH		DIFF.	PROB.
CR-NR	+2.85	—1.40	4.25	0.0124
CF-NF	+2.02	—1.48	3.50	0.0394
CR-CF	+2.85	+2.02	0.83	0.1294
NR-NF	—1.40	—1.48	0.08	0.8542
CF-NR	+2.02	—1.40	3.42	0.0494
CR-NF	+2.85	—1.48	4.33	0.0118

water on both vitamin-rich and vitamin-free diets. The average for the four experiments showed 4.25% greater growth in conditioned than in non-conditioned water on the vitamin-rich diet, with a statistical probability of 0.0124. The same comparison between series on the vitamin-free diet showed 3.50% greater growth in conditioned water. The statistical probability is 0.0394.

Taken together these experiments show then, a lack of difference in growth associated with a difference in vitamin content of the diets, and significantly better growth of the series in conditioned water than in non-conditioned water at the end of 20 days.

Analysis of the survival data after extended periods of experimentation gives a slightly different picture of the

effects of various procedures. When the data for the preliminary and the later experiments are considered together, the results indicate a favorable effect of vitamins on the survival of the fishes. However, the same criticism must be applied here that was used before in discussing growth, namely, that the difference in the composition of the diets, particularly the inclusion of fresh animal matter in the

TABLE 4
Survival data

EXPERI- MENT	DAYS	CONDITIONED				NON-CONDITIONED			
		Vitamin-rich CR		Vitamin-free CF		Vitamin-rich NR		Vitamin-free NF	
		A ¹	B ²	A	B	A	B	A	B
11	20	10	10	10	10	10	8	10	9
	40	10	10	10	7	10	8	9	8
	60	10	10	9	6	10	7	9	5
12	20	9	9	10	9	10	9	10	9
	40	9	8	10	8	10	5	10	4
14	20	10	10	10	10	10	10	10	10
	40	9	9	10	8	10	8	9	4
	60	8	6	10	6	10	5	9	0
15	20	12	12	12	12	12	10	12	12
	40	12	12	12	12	12	10	12	11
	60	12	10	12	10	12	7	12	6
	100	11	7	12	7	12	4	12	0
	140	11	7	12	6	12	2	12	0

¹ Initial number of fishes.

² Number of fishes surviving at end of experiment.

vitamin-rich diets in the preliminary experiments might have caused the effects noted rather than vitamins alone.

The survival data for the later, more critical experiments are given in table 4 as the numbers of fishes surviving in each series at the 20-day intervals of experimentation. The survival of fishes was also calculated as the percentage of possible time lived by each fish. Fishes living to the end of an experiment were counted as living 100% of the time;

30 days of a 40-day experiment, 75%; 30 days of a 60-day experiment, 50%, etc. These results are given graphically for each experiment separately in figure 2 and with statistical analyses of the averages for all of the fishes under the different experimental conditions in table 5. Figure 2 is self-explanatory. In table 5 not all of the forty-two possible pairs of fishes were represented in each comparison, due to

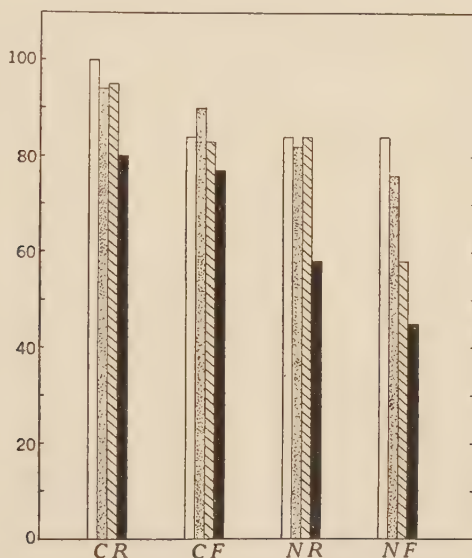


Fig. 2 Survival results in experiments 11, 12, 14 and 15. The vertical axis gives the percentage of time survived. Each column represents the average survival of the fishes in one series, expressed as a percentage of the total time of the experiment. (Survival for the whole time of an experiment was 100%.) Symbols: outlined, experiment 11; stippled, experiment 12; diagonal lines, experiment 14; solid black, experiment 15. CR, conditioned water, vitamin-rich diet; CF, conditioned water, vitamin-free diet; NR, non-conditioned water, vitamin-rich diet; NF, non-conditioned water, vitamin-free diet.

the accidental loss of several fishes. For this reason the percentage survival for any one series differs slightly in the different comparisons. The analysis shows without a doubt the favorable effect of conditioned water on survival, whether the fishes were fed on the vitamin-rich or the vitamin-free diet. The effect of vitamins is statistically not so significant,

since the differences between survival on the vitamin-rich and vitamin-free diets had probabilities of 0.0574 and 0.0718 for comparisons made on fishes isolated in the conditioned and non-conditioned water respectively. However, these values tend to show a difference in the survival of fishes on the two diets in favor of the vitamin-rich, and more cases would probably serve to make this difference significant statistically. As they stand, the differences of 9.5% and 11.1% longer survival on the vitamin-rich diet must be taken as indicative only. The fifth comparison shows an insignificant difference between the survival of fishes in conditioned water on the vitamin-free diet and in non-conditioned water

TABLE 5

Statistical comparison of survival data in experiments 11, 12, 14 and 15

COMPARISON OF SERIES	NUMBER IN EACH SERIES	PER CENT OF TIME SURVIVED ¹		DIFF.	PROB.
CR-NR	38	92.0	74.9	17.1	0.0052
CF-NF	39	82.4	63.6	18.8	0.0006
CR-CF	37	91.8	82.3	9.5	0.0574
NR-NF	40	75.6	64.5	11.1	0.0718
CF-NR	41	83.2	75.5	7.8	0.1936
CR-NF	36	91.5	65.4	26.1	0.0000

¹ Each figure represents the average survival of each series of fishes, expressed as a percentage of the total time of the experiment. (Survival for the whole time of the experiment was 100%.)

on the vitamin-rich diet (83.2% vs. 75.5%), and the last gives a very great statistical significance between the conditioned water vitamin-rich series and the non-conditioned water vitamin-free series (91.5% vs. 65.4%).

The mean survival values for series CR (92%), CF (83%), NR (75%) and NF (65%) reveal a gradation in favorable conditions which affect the length of life of the fishes. With a combination of a vitamin-rich diet and a conditioned water medium the best survival results are obtained. With conditioned water alone the survival results are not so good, but they are better than with vitamins alone. With neither vitamins nor conditioned water present the fishes survive very poorly.

A study of growth data (table 3) and survival data (table 5) reveals interesting similarities and differences in the results of the statistical comparisons made. In both growth and survival the effect of conditioned water is statistically significant. Comparisons of the effect of the diets, however, show an indicated effect of vitamins on survival but not on growth. A curious situation results from these relationships with regard to the comparison between fishes in conditioned water on the vitamin-free diet, and in non-conditioned water on the vitamin-rich diet (CF:NR). This comparison in the case of survival shows a difference of 7.8% which is statistically insignificant; this would indicate that conditioned water alone and vitamins in the diet are about equal in their effect on survival. This does not mean, however, that the effect of conditioned water is a vitamin effect, since an additional effect of vitamins is shown in the greater survival of fishes in conditioned water getting the vitamin-rich diet. In the case of growth, the comparison between series CF and NR gives a difference of 3.42% greater growth in conditioned water on the vitamin-free diet than in non-conditioned water on the vitamin-rich diet. This value is statistically significant and indicates that conditioned water is much more important as far as growth is concerned than are added vitamins in the diet. The contradictory results of growth and survival in the comparison between series CF and NR are due to the fact that, while the differences in survival between conditioned water and non-conditioned water series on both types of diets are statistically significant, the differences are not great enough so that the series having the lowest survival in conditioned water is significantly greater than the series having the highest survival in non-conditioned water. On the other hand, since in these experiments vitamins seem to have no effect on growth, the differences between both the vitamin-free and vitamin-rich series in conditioned water and the vitamin-rich series in non-conditioned water are statistically significant.

RAT ASSAY OF VITAMINS IN FISH-CONDITIONED WATER

In order to make an additional test for vitamins in fish-conditioned water a feeding experiment was carried out on white rats after first doing a preliminary experiment to establish the technique. Twenty-eight female rats between 20 and 30 days of age⁵ were distributed among seven groups in two series with litter mates placed in corresponding positions in the series in most cases. The rats were placed two to a cage, in wire cages with raised wire bottoms to prevent access to excreta. The trays of the cages were lined with paper towelling and both cages and trays were cleaned two or three times a week.

The experiment was planned to test for water soluble vitamins (B and G) and for fat soluble vitamins (A and D), separately, both in conditioned water and in a concentrated fish protein extract which had been found to promote growth of isolated goldfishes when used in great dilutions. Although the vitamin assay of this protein material does not add to the analysis of the factors dealt with in this problem, it seemed advisable to include the two groups on protein extract as an analysis of other factors dealt with in similar experiments on fish growth carried out in this laboratory (Allee et al., '36).

The following basal diet (Macy et al., '27) was fed to all of the groups: casein (vitamin-free), 18%; dextrin, 76%; salts (Osborne and Mendel), 4%; agar agar, 2%.

The vitamin supplements and types of liquid used to moisten the diets are given in table 6. Dried yeast was used as the source of vitamins B and G and haliver oil for A and D. The basal diet for each two rats was weighed daily and 1 drop of haliver oil was added for each two rats on diets 1, 2, 3 and 7, and 0.5 gm. yeast per rat for diets 4, 5, 6 and 7 (0.5 gm. per 10 gm. of dry diet for amounts over 20 gm). In addition, after the fourteenth day, each rat in the series getting oil was given 1 drop of haliver oil by pipette every other day.

⁵Dr. Z. Wallen-Lawrence of the Department of Biochemistry very kindly furnished litters of known ages for this experiment.

The dry diets were moistened with twice the amount of liquid (of appropriate source) as the weight of dry mixture. After 24 days this was increased to three times the dry weight. The food remaining uneaten by the next day was dried in an oven for 24 hours and weighed. In this way the food consumption of each two rats could be determined.

The concentrated fish-conditioned water used for moistening the diets had a conditioning coefficient of 296 (about

TABLE 6
Supplements to rat diets

RAT DIET	VITAMINS				LIQUID		
	A	D	B	G	S.P.W. ¹	Fish-conditioned S.P.W.	Fish protein extract
Series I							
1 ²	+	+	..	..	+	..	..
2	+	+	..	..	..	+	..
3	+	+	..	..	..	..	+
Series II							
4 ²	..	..	+	+	+	..	..
5	..	..	+	+	..	+	..
6	..	..	+	+	..	..	+
Positive control							
7	+	+	+	+	+	..	..

¹ Synthetic pond water.

² Negative control.

thirteen times the concentration usually used in fish experiments). The conditioning fishes were fed on raw liver or oatmeal for 3 hours daily. Liver was fed 4 days a week and oatmeal twice a week. The appearance of the water was noticeably different following the feeding of liver or oatmeal; the day following an oatmeal feeding the water was decidedly murky from faeces and regurgitated food particles. The water was sieved as in experiment 15 before it was used in the rat diets.

The protein extract was prepared by Dr. R. B. Oesting from slime secreted by fish on the surface of their bodies

when treated with about a 0.1% solution of sodium carbonate. The extract so prepared was autoclaved and stored in the ice box. A new preparation was made about every 10 days to 2 weeks. Dilutions of 1:100 of similar extracts had been found to give positive results in growth stimulation of small goldfishes (Allee et al., '36).

Non-conditioned synthetic pond water was used in the control diets and in all of the water bottles for drinking water except in the conditioned water series, in which conditioned water was used for drinking water also.

The rats were weighed every 3 or 4 days and individual growth curves were plotted for each rat. Figure 3 shows the weekly gain in weight for each group, averaged for four rats. The experiment was terminated at the end of 10 weeks.

In series I (diets 1, 2 and 3, without yeast) there was a slight gain for about 1 week on all three diets, with a continued slight gain for the second week on the diet getting fish-conditioned water (diet 2). During the third week deaths occurred on diets 1 and 3, and during the fourth and fifth weeks on each diet in the series. The characteristic symptoms before death were inactivity and loss of appetite, followed by inability to maintain balance and finally paralysis. From the fourth week on, both the negative controls and the group getting fish-conditioned water maintained their weight at a fairly constant level, indicating the presence of small amounts of vitamin B in the basal diet. The slightly higher initial growth of the rats on diet 2 (on fish-conditioned water) and their maintenance at a slightly higher level for several weeks, indicates a small amount of vitamin B or G or both present in the fish-conditioned water. However, the deaths on this diet earlier in the experiment are evidence that at least in the quantities used, the amount must be very low.

In series II (diets 4, 5 and 6, without haliver oil) there was a similar relationship between the group getting conditioned water and its negative control. The rats with the conditioned water supplement reached an appreciably higher

average weight and maintained themselves at this higher level. One rat in this group survived the others of the series by 3 weeks. These results probably indicate the presence of small amounts of vitamin A in the fish-conditioned water, although the amount was too small to prevent the appearance of xerophthalmia, as evidenced by exudations around

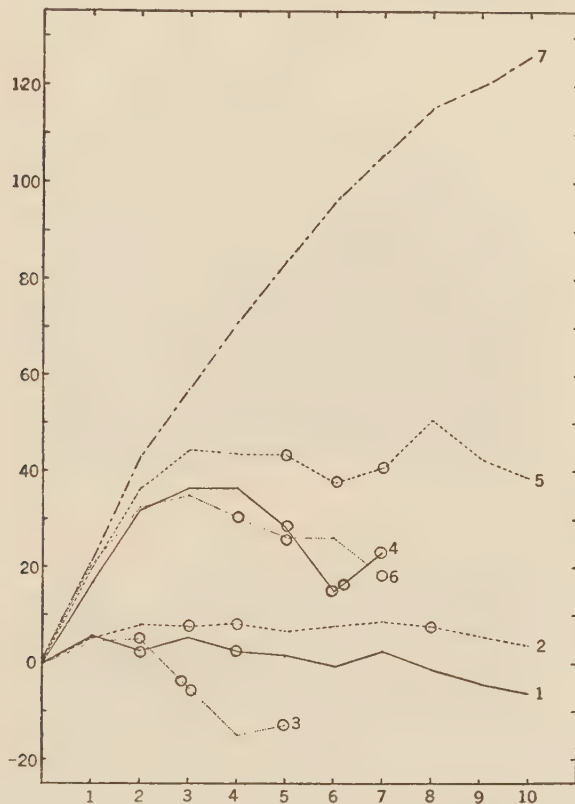


Fig. 3 Growth of rats in assay for vitamins in fish-conditioned water and fish protein extract. Time in weeks is plotted on the horizontal axis and gain in weight in grams on the vertical axis. The curves show the average weekly gain in weight of rats on the following diets: 1, 2, 3, without vitamins B and G (no yeast); 4, 5, 6, without vitamins A and D (no haliver oil); 7, positive control with vitamins B, G, A and D supplied in yeast and haliver oil. Diets 1, 4 and 7 were supplemented with non-conditioned synthetic pond water; 2 and 5 with fish-conditioned synthetic pond water; 3 and 6 with fish protein extract. Each curve represents an average for four animals at the start, with the exception of the curve for diet 6, in which only three animals are included. A circle indicates that a death occurred during the following week.

the eyes about the fourth week. About the same time respiratory infections causing the rats to cough occurred on all three diets, and deaths occurred in the sixth to eighth week.

The rapid gain in weight and freedom from infection on diet 7 is evidence of the adequacy of the diet serving as positive control for both the yeast and haliver oil series. There

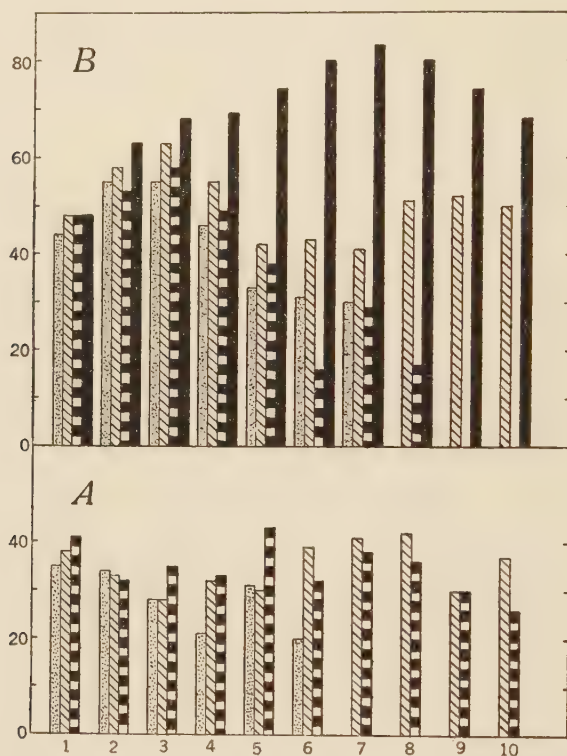


Fig. 4 Food consumed by rats in vitamin assay. Average amount of basal diet consumed per week: A, by rats on diets 1, 2, 3; B, by rats on diets 4, 5, 6 and 7. Diets 2 and 5 contained fish-conditioned water. Time in weeks is plotted on the horizontal axis and amount of diet consumed in grams on the vertical axis. Symbols: stippled, diets 3 and 6; diagonal lines, diets 2 and 5; interrupted black, diets 1 and 4; solid black, diet 7.

is no evidence for the presence of any vitamin in the fish protein extract.

The average amount of food eaten per week by rats on each of the experimental diets is shown in figure 4. The

occurrence of deaths makes the weekly averages less reliable toward the end of the experimental period, but the greater consumption of food to which fish-conditioned water had been added seems to correspond with the higher average weight attained on these two diets.

The inability of rats to attain normal growth on the diets supplemented with conditioned water in the amounts used, does not necessarily mean that fishes living in the water could not get an adequate supply from it. The rat experiment was merely performed as an assay of the vitamins present in terms of the effect on the growth of a standard animal. This experiment does not test for vitamin C in the water since rats are not suitable for such a test. However, McCay and Dilley ('27) have shown that the addition of vitamin C to a synthetic diet has no effect on growth of trout, and it is not probable that the effect of conditioned water is due to the presence of this vitamin.

DISCUSSION

A convincing positive solution of the problem involves in the first place the necessity of demonstrating that fishes need vitamins in order to grow, or grow better with vitamins than without them in non-conditioned (uncontaminated) water. If this were demonstrated, and granting that the usual stimulating effect of conditioning was present, then fishes fed on the vitamin-rich and vitamin-free diets in conditioned water would have two possible relationships. They could either grow better on the vitamin-rich diet, indicating that conditioned water qualitatively or quantitatively lacks vitamins, or they could grow equally well. If they grew equally well there could still be two interpretations as to the cause of the lack of difference. Either the conditioned water provided sufficient vitamins to compensate for the lack of them in the vitamin-free diet, or conditioned water itself contained some distinct growth promoting power which evoked the highest response possible, so that fishes growing at their highest rate could grow no more with vitamins added in the diet.

Part of the difficulty in analyzing our results lies in the fact that our experiments have not demonstrated that the presence of vitamins in the diet actually makes any difference in the growth of goldfishes in non-conditioned water, although there was an indicated difference in survival. It is true that after the first few weeks the fishes in non-conditioned water did not eat well, and as has been stated this fact in itself may be the result of a dietary deficiency of some kind such as a lack of raw meat. It is possible that the fishes were eating so little that this was not an adequate test of the effect of the two diets on growth. Apparently they ate enough during the first weeks of the experiment to account for the difference in survival. This difference in survival is the only evidence from series in non-conditioned water of a vitamin effect in the diets.

When we turn to the series in conditioned water, we find the usual evidence of a growth stimulating factor. In the first weeks of the experiment when all of the fishes were eating their food, the fishes in conditioned water grew more than those in non-conditioned water. Supposing for a moment that this were a vitamin effect, it would be hard to explain, particularly in experiment 15, why there should be such decided improvement in the growth of fishes on the vitamin-free diet in conditioned water over that of fishes on the same diet in non-conditioned water (CF:NF), when an abundance of vitamins in the vitamin-rich diet in non-conditioned water had produced no such effect (NR:NF). Moreover, the great difference in growth between fishes exposed to the conditioning factor alone and those with the vitamin factors alone (table 3, CF:NR) indicates that the conditioning effect must be more than a vitamin effect.

The possibility that non-conditioned synthetic pond water is actually harmful, either because of the presence of some injurious substance or because of an unfavorable inorganic composition, and that biological conditioning in some way either removes the toxic effects or supplies something that is necessary and thus produces the favorable results we have

found, does not seem probable in view of the fact that in two of the preliminary experiments and in experiment 14 fishes grew well in non-conditioned water. While these fishes may have been in unusually good condition for growth, the fact that they grew is evidence that non-conditioned water is not essentially harmful. Other experiments carried out in this laboratory in which a particularly favorable diet was fed to the assay fishes also resulted in excellent growth in similar non-conditioned water.

A suitable explanation at least for a large part of the growth promoting effect of conditioned water is that given by Allee, Oesting and Hoskins ('36), namely, that the conditioned water contains small particles of faeces or regurgitated food contributed to the water by the conditioning fishes, and that these particles which cannot be removed by sieving, furnish an additional source of food for the assay fishes in conditioned water. These fishes are thus able to supplement their usual limited feeding period by feeding throughout the day on the food particles in conditioned water and naturally grow more than those in non-conditioned water which do not have this opportunity.

As for the fact that the fishes grew equally well on both diets in conditioned water, there are two possible explanations. The suggestion made above, that the fishes might be growing so much due to the conditioning factor that an addition of vitamins to the diet could elicit no further response, is not applicable here since the fishes did not grow at their highest rate possible, but rather scarcely grew at all after 60 days. We must either suppose that goldfish do not need vitamins in order to grow, or that they get enough vitamins from the food particles introduced by conditioning fishes to compensate for the lack of vitamins in the vitamin-free diet. It seems possible that food particles large enough to have actual caloric value might easily carry vitamins with them. However, the experiment with rats indicates that if there are vitamins in the conditioned water, they must be present in very low concentrations.

The explanation that fishes do not depend on vitamins for growth is contrary to the results of other workers in this field, particularly those of Jewell, Schneberger and Ross ('33). In an experiment of about 80 days' duration involving 200 goldfishes, they report 2.7% better growth on their diet including vitamin D, as compared with growth on the diet lacking vitamin D; unfortunately, statistical comparisons are not given nor can statistical values be calculated from their published data. Moreover, there was 11% better survival on the vitamin D-free diet. In our experiment 15 at 80 days there was 3.3% greater growth on the vitamin-free than on the vitamin-rich diet, yet this difference was statistically insignificant. Our experiment involved only twenty-four fishes and it is probable that with the larger number of fishes used in the above experiment a difference of 2.7% would prove to be significant. These authors admit that their data for vitamins B and G are much less conclusive.

In our experiments the explanation that goldfish do not require vitamins A, B, C, D or G for growth, at least for a limited time, seems the best explanation of the failure to produce a difference in the growth of fishes fed on the two diets in conditioned water. The alternative explanation, that vitamins are carried in the food particles in conditioned water, which compensate for the lack of vitamins in the vitamin-free diet does not seem justified in view of the fact that an effect of the vitamins presented was not demonstrated in non-conditioned water insofar as growth is concerned. We can suppose that the fishes in non-conditioned water were not getting enough food during their limited daily feeding period to permit growth, and that with food available for longer periods a vitamin effect on growth might have shown up during the first weeks when the fishes were still eating their daily rations. As it is, we have presumptive evidence that the addition of vitamins to the diet did suffice to increase the length of survival of the fishes getting this diet beyond that of fishes on the vitamin-free diet. The fact that fishes survived longer on vitamin-rich than on vitamin-free diets

in conditioned as well as in non-conditioned water, indicates that this was probably an effect of vitamins which was not compensated for by conditioned water. From this and from other considerations it seems evident that the conditioning effect on both growth and survival is not primarily a vitamin effect.

As to which vitamin or vitamins are responsible for the survival effect, we have not been concerned up to the present time. No attempt was made to provide 'factor H' in isolated form. It is possible that the cessation of growth of fishes after extended periods on synthetic diets with vitamins A, B, C, D and G supplied, may be due to the lack of this factor, the presence of which McCay and Dilley ('27) postulated in raw meat. In the longest experiment (140 days) there was very little growth after 60 days. Since the conditioning fishes as well as the experimental fishes received no raw meat during this time, there was no 'factor H' available either in the diet or in the conditioned water. The conditioning fishes in the other experiments received raw liver in their diet and it is possible that 'factor H' was present in the conditioned water. However, none of the other experiments lasted longer than 60 days, and there does not seem to be any correlation between the results of growth during the first 60 days of experimentation and the type of diet fed to the conditioning fishes. A study of the effect of various kinds of diets fed to the conditioning fishes on the growth stimulating power of conditioned water should prove interesting.

SUMMARY AND CONCLUSIONS

1. To test the possibility that the growth promoting effect of conditioned water might be due to vitamins excreted by the conditioning fishes, assay fishes were fed for 2 to 3 hours daily: a) a vitamin-rich diet in homotypically conditioned water and b) the same diet in non-conditioned water; c) a vitamin-free diet in conditioned water and d) the same diet in non-conditioned water.

2. The synthetic diet used consisted of casein, starch and salts, with haliver oil, yeast and lemon juice added for the vitamin-rich diet, which was also fed to the conditioning fishes.

3. Fishes grew significantly better in conditioned than in non-conditioned water whether they were fed on the vitamin-rich or the vitamin-free diet.

4. In conditioned water the fishes grew equally well on both diets.

5. In non-conditioned water on both vitamin-rich and vitamin-free diets, the fishes failed to grow. There was no significant difference in the amount of failure.

6. The effect of vitamins showed in an indicated better survival of the fishes on the vitamin-rich than on the vitamin-free diet in both kinds of water.

7. An assay of conditioned water fed to rats on vitamin deficient diets showed that vitamins A, B, D and G if present, were present in very low concentrations.

8. Better growth of fishes in conditioned than in non-conditioned water has recently been explained (Allee et al., '36) as largely a result of the additional food value of particles of faeces or regurgitated food which are contributed to the water by the conditioning fishes and are too small to be removed by sieving. Since assay fishes were fed for a limited time only, each day, the fishes in non-conditioned water were at a disadvantage in lacking this additional food supply.

9. The lack of difference in growth of fishes fed on vitamin-rich and vitamin-free diets in conditioned water could either be explained by the fact that goldfish do not need vitamins in order to grow, or that they received vitamins from the conditioned water. Other workers have demonstrated that the growth of goldfish is probably affected by one, if not more, vitamins. It is, moreover, reasonable to assume that if there are particles present in conditioned water of such size that they can furnish actual food value, they might also carry a sufficient quantity of vitamins with them to compensate for the lack of vitamins in the vitamin-free diet. Nevertheless,

the explanation that goldfish can grow at least for a limited time without vitamins seems the more logical, in view of the fact that these experiments did not demonstrate that vitamins made any difference in the growth of fishes in non-conditioned (uncontaminated) water.

10. The fact that fishes apparently survived longer on vitamin-rich than on vitamin-free diets in conditioned as well as in non-conditioned water indicates that there is a survival effect of vitamins in the diets, which is not masked by the presence of conditioned water.

11. From these lines of reasoning it seems evident that vitamins are not the essential factor in the growth stimulating power of conditioned water.

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